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Prevalence of periodontal bacteria in saliva of Kuwaiti children at different age groups

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Summary *Aggregatibacter* (formerly *Actinobacillus*) *actinomycetemcomitans*, *Tannerella forsythensis* and *Porphyromonas gingivalis* and to a lesser extent *Prevotella intermedia* and *Prevotella nigrescens*, are Gram-negative species that are associated with destructive periodontitis. Studies from different parts of the world have shown variable detection rates of periodontal organisms. Hardly any data exist on their carriage in children living in the Middle East. This study was designed to determine the detection of these species in the oral cavity of 240 generally healthy Kuwaiti children, divided into five age groups: <6 years ($n = 40$), 6—9 years ($n = 60$), 10—12 years ($n = 40$), 13—15 years ($n = 40$) and 16—18 years ($n = 60$). Saliva was used as the microbiological specimen, and the samples were analyzed by molecular methods using multiplex PCR. A total of 185 (77.1%) of the 240 children were colonized by at least one of the target periodontal bacteria. In all age groups, *P. nigrescens* was the most prominent and detected in saliva of 15%, 32%, 63%, 50%, and 47% of the children at the five age groups, respectively. *P. gingivalis* was detected only occasionally. Only few pathogens were found before the permanent dentition, i.e. at the age of <6 years. The highest carriage rates were from the groups between 6 and 15 years of age. The salivary carriage of the pathogens was essentially similar in the age groups of 10—12 years and 13—15 years. In conclusion, except for *P. gingivalis*, the

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examined periodontal pathogens are relatively common findings in Kuwaiti children and colonize the oral cavity from childhood onwards.

Introduction

Periodontal diseases are a group of chronic infections that destroys tissues surrounding and supporting the teeth. Approximately a dozen oral bacterial species are associated with periodontitis. However, to date, the most convincing data implicate three microorganisms as major etiological agents in the initiation and progression of periodontitis. They are *Aggregatibacter* (formerly *Actinobacillus*) *actinomycetemcomitans*, *Tannerella forsythensis* and *Porphyromonas gingivalis* [1,2]. *A. actinomycetemcomitans* is frequently associated with early-onset periodontitis, especially localized aggressive periodontitis [3,4]; whereas *P. gingivalis* is associated with chronic periodontitis [2]. Moderately strong evidence has been demonstrated for other bacteria isolated from subgingival microbiota, of which *Prevotella intermedia* and *Prevotella nigrescens* may have a greater relevance in periodontal diseases [5]. However, the oral colonization of these organisms is not particularly limited to periodontally diseased sites. *T. forsythensis* and *P. nigrescens*, for instance, have been found at gingival margin and in the sulcus of periodontally healthy sites [5,6]. Unlike most oral anaerobes with a gradually increasing prevalence with age, true periodontal pathogens generally may not colonize the oral cavity in early childhood but later in life, if ever [7].

Early childhood years are the critical period for the acquisition of certain bacteria, and close household contacts positive for periodontal pathogens are the main source of acquisition for infants and children [6,8]. It is of particular interest to investigate the early colonization of children with periodontal pathogens, since their presence could identify a patient as a carrier or being at a risk of developing periodontitis in adolescence [8,9].

The development of techniques in molecular biology, aimed at the detection of bacterial pathogens, has allowed not only the acquisition of knowledge in microbial genetics but has also set the bases for the development of improved diagnostic techniques. Polymerase chain reaction (PCR) has emerged as the most powerful tool for the amplification of genes and their RNA transcripts [2]. It is now widely accepted that the PCR methods provide a more sensitive means of detection of putative bacterial species, as compared to conventional culture techniques [10,11]. It is quick, relatively simple, and able to detect low numbers of bacterial species with detection limits of as few as 25—100 cells [6]. A PCR assay is also suitable for the detection of periodontal pathogens, especially in subgingival plaque in children where there are a limited number of pathogens present [6,8].

Since there are no data on carriage rates of periodontal pathogens among young individuals of Arabic origin, we undertook the task of studying the carriage rates of *A. actinomycetemcomitans*, *T. forsythensis*, *P. gingivalis*, *P. intermedia*, and *P. nigrescens* among children and adolescents in Kuwait.

Materials and methods

Study subjects

The subjects (120 boys and 120 girls) were enrolled into the study at the School Health Program, Salmiya, at primary and secondary schools in Jabriya and Hawally regional governorates as well as undergraduate dental students in their first year of the Dental School in Jabriya. The study was conducted over a period of 2 years, from January 2003 to December 2005. These children were stratified into five age groups, <6 years (group A), 6—9 years (group B), 10—12 years (group C), 13—15 years (group D), and 16—18 years (group E). Subjects receiving or those that have received antimicrobial therapy in the last 3 months prior to our visit were excluded from the study. Informed consents from the parents of the children were obtained after thorough explanation of the aims and objectives of the study and the project was approved by the Faculty's Ethics Committee.

Specimen collection

Salivary samples were collected from 40 children in group A, 60 children in group B, 40 children in group C, 40 children in group D, and 60 children in group E. Paraffin-stimulated saliva was expectorated into medical cups and then a sterile cotton wool swab

sample was collected from cheeks, palate and dorsum of tongue and pooled with the salivary sample. The samples were divided into two Eppendorf tubes (one for multiplex PCR and another for further use) and immediately transported in ice to the Anaerobe Reference Laboratory, Department of Microbiology, Faculty of Medicine, Kuwait University. The specimens were processed within 10 min of arrival in the laboratory, and the unused ones stored at −70 °C until processed.

Bacterial DNA extraction

Bacterial DNA was extracted from the saliva samples, treated with cation-chelating resin [12], using a commercially available QIA amp DNA blood mini kit (QIAGEN Inc., Valencia, USA), according to the manufacturer's protocol. Briefly, to a 80 μl of the sample in PBS, was added 120 μl of ATL buffer provided in the kit and 20 μl of proteinase K, in a micro-centrifuge tube. The mixture was pulse-vortexed for 15 s, and incubated at 56 °C for 15 min, and then pulse-centrifuged to bring down any droplets on the lid of the tube. 200 μl of ATL buffer was again added to the tube, pulse-vortexed for 15 s, and then incubated at 70 °C for 10 min. The tube was then spun briefly for 15 s and 200 μl of ethanol (96%, v/v) added and mixed thoroughly. The content was transferred into QIAamp spin column (provided in the kit) and centrifuged at 8,000 rpm for 1 min. Filtrate was discarded and 500 μl of buffer AW1 (wash buffer) added to the pellet, mixed and centrifuged at 8,000 rpm for 1 min and the supernatant discarded. Again, 500 μl of buffer AW2 was added to the spin column and centrifuged at 14,000 rpm for 1 min and the filtrate discarded. The spin column was then placed in a micro-centrifuge tube and 200 μl of buffer AE (elution buffer) was added and incubated at room temperature for 5 min. After incubation, the tube was centrifuged at 8,000 rpm for 1 min. The eluted DNA in the micro-centrifuge tubes was then stored at −20 °C until further use.

Bacterial detection by multiplex PCR

The eluted supernatant containing bacterial DNA served as template for the PCR amplification using the method previously described by Ashimoto et al. [11]. Each amplification reaction was performed in a total volume of 50 μl (44 μl of Master mixture and 6 μl of DNA template). Master mixture contained 5 μl 10× PCR Gold Buffer, 5 μl 25 mM $MgCl_2$, 4 μl dNTP mix, 2 μl of *P. gingivalis* and 2 μl of *T. forsythensis* primer (forward and reverse primers mixed) and 0.25 μl of Amplitaq Gold DNA polymerase enzyme. Also *A. actinomycetemcomitans*, *P. intermedia*, and *P. nigrescens* were done as above except that the appropriate primers were added. Reference strains *A. actinomycetemcomitans* (ATCC 43718, ATCC 29523, ATCC 29524), *T. forsythensis* (ATCC 43037), *P. gingivalis* (ATCC 33277), *P. nigrescens* (ATCC 25261, ATCC 33563) and *P. intermedia* (ATCC 2566) were used as positive controls. Table 1 lists the PCR primers used in this study. Each PCR primer pair was tested against samples containing the single target organism alone and samples containing mixed target organisms. The cycling parameters were as follows: 1 cycle of 95 °C for 10 min, 38 cycles of 95 °C for 1 min, 60 °C for 1 min, 72 °C for 2 min, 1 cycle of 72 °C for 10 min and 4 °C infinity. Since each clinical samples yielded an amplicon from at least one tested organism the possibility of PCR inhibitors in the extracted DNA samples was ruled out. Post-multiplex PCR gel electrophoresis was performed by mixing an aliquot of 10 μl of the amplified samples with 3 μl gel loading dye and electrophoretically ran in a 2.5% agarose in TAE buffer for 115 min at 83 V. The amplification products were visualized and photographed under UV light after 1 h of ethidium bromide staining. Typical bands of the organisms are shown in Fig. 1.

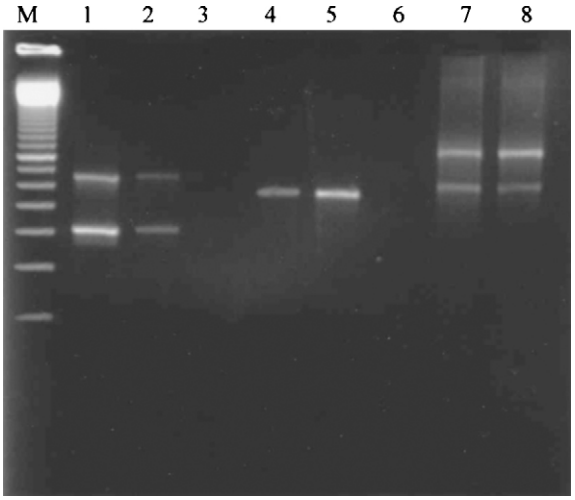

Figure 1 PCR amplification bands: M, 100 bp ladder; Lane 1, positive control of *P. gingivalis* ATCC 33277 (404 bp) and *T. forsythensis* ATCC 43037 (641 bp); Lane 2, positive salivary sample for *P. gingivalis* and *T. forsythensis*; Lane 3, negative control; Lane 4, positive control, *A. actinomycetemcomitans* ATCC 29523 (557 bp); Lane 5, positive salivary sample showing the presence of A. *actinomycetemcomitans*; Lane 6, negative control; Lane 7, positive control for *P. intermedia* ATCC 2566 (575 bp) and *P. nigrescens* ATCC 25261 (804 bp); Lane 8, positive salivary sample showing the presence of *P. intermedia* and *P. nigrescens*.

Table 1 Species-specific and ubiquitous primers for PCR.

Primer pairs (5′—3′)	Base position (amplicon length in bp)
A. actinomycetemcomitans AAA CCC ATC TCT GAG TTC TTC TTC ATG CCA ACT TGA CGT TAA AT	478—1034 (557)
T. forsythensis GCG TAT GTA ACC TGC CCG CA TGC TTC AGT GTC AGT TAT ACC T	120—760 (641)
P. gingivalis AGG CAG CTT GCC ATA CTG CG ACT GTT AGC AAC TAC CGA TGT	729—1132 (404)
P. nigrescens ATG AAA CAA AGG TTT TCC GGT AAG CCC ACG TCT CTG TGG GCT GCG A	219—1022 (804)
P. intermedia TTT GTT GGG GAG TAA AGC GGG TCA ACA TCT CTG TAT CCT GCG T	458—1032 (575)

Results

Out of 240 children studied, 185 (77.1%) were positive for the targeted periodontal pathogens. In descending order, the overall occurrence rates of these pathogens were as follows: 41% for *P. nigrescens*, 20% for *A. actinomycetemcomitans*, 10% for *T. forsythensis*, 8% for *P. intermedia*, and 2% for *P. gingivalis*. By age group, at least one or more periodontal pathogens were carried in saliva by 10 (25%) of the 40 children aged <6 years (group A), 46 (77%) of the 60 children aged 6—9 years (B); 40 (100%) of the 40 aged 10—12 years (C); 40 (100%) of the 40 children aged 13—15 years (D), and 49 (81.7%) of the 60 adolescents aged 16—18 years (E).

As shown in Table 2, of the 40 children investigated in group A, the predominant isolate was *P. nigrescens* (15%) followed by *A. actinomycetemcomitans* (7.5%); no *P. gingivalis* or *T. forsythensis* was isolated. Only one child carried *P. intermedia*. The commonest in age group B were *A. actinomycetemcomitans* and *P. nigrescens*, each isolated from the saliva of 31.7% of children. *P. gingivalis* was isolated in only one child.

In age group C, all children carried at least one periodontal pathogen in the saliva and 15 children carried two or pathogens per child. The predominant pathogen was *P. nigrescens* (62.5%) followed by *A. actinomycetemcomitans* (27.5%) and *T. forsythensis* (17.5%). Again *P. gingivalis* was isolated in only 1 (2.5%) of the children in this age group.

All the children in group D carried at least one pathogen and *P. nigrescens* was also the commonly detected pathogen with a prevalence of 50%; followed by *T. forsythensis* and *A. actinomycetemcomitans* (20% each). Only 10% of children harbored *P. intermedia* in this age group; while *P. gingivalis* was absent in all. In group E children, *P. nigrescens* was again the most prominent periodontal pathogen which was isolated in 46.7% of children followed by *T. forsythensis* and *A. actinomycetemcomitans* in 17.5% and 11.7%, respectively. *P. gingivalis* was found in just 3.3%.

Discussion

So far, there are only a few prospective longitudinal data on the prevalence of periodontal pathogens in the oral cavity of healthy children. As shown in the present study, Kuwaiti children harbored most of these periodontal pathogens before the age of 18 years. Also, with the exception of *P. gingivalis*, the carriage of periodontal pathogens is common in childhood after the age of 6 years, attaining adult carriage rate by the age of 9—12 years [13]. Only about 22.9% of the children were free of these target periodontal bacteria and these were mainly children below the age of 6 years.

Many studies have reported the presence of periodontal pathogens in the oral cavity of both healthy and diseased individuals (5,14). Some of these reports indicate prevalence of *A. actinomycetemcomitans* is low in young children and increases with age, but on the contrary, our results showed that its prevalence was highest in age between 6 and 9 years and thereafter steadily decreased with age, down to the lowest in the age group 16—18 years. Our finding is supported by an earlier study conducted by Yuan et al. [14] among school children in Taiwan where they found the prevalence of *A. actinomycetemcomitans* was highest in age group 7—12 year olds and thereafter declined in adult control group. In this Yuan's study, it was reasoned that following adolescents, *A. actinomycetemcomitans* might be gradually overcome by immunological factors. The reason why the prevalence of *A. actinomycetemcomitans* declined as age increased in our subjects may be due to the fact that other

Table 2 Carriage rates of target periodontal pathogens in the salivary samples of children at various age groups.

Stratified age group (no. of subjects)	*A. actinomycetemcomitans* (%)	*P. gingivalis* (%)	*T. forsythensis* (%)	*P. intermedia* (%)	*P. nigrescens* (%)
<6 years (40)	7.5	0	0	2.5	15
6–9 years (60)	31.7	1.7	5	6.7	31.7
10–12 years (40)	27.5	2.5	17.5	15	62.5
13–15 years (40)	20	0	20	10	50
16–18 years (60)	11.7	3.3	11.7	8.3	46.7

teeth in the permanent dentition are usually protected from *A. actinomycetemcomitans* challenge because of the host's immune system which provides increasingly stronger protection with age and the periodontal ecosystem of the teeth that disfavors *A. actinomycetemcomitans* [14]. It is also possible that the declining prevalence of this organism in older children may be due to displacement by other periodontal bacteria competing for the same niche. A further longitudinal study is required to prove this hypothesis.

The pathogenic potential of *P. nigrescens* and *P. intermedia* appears to be different. It, indeed, seems that while *P. nigrescens* can be regarded as a common member of the oral microbiota, *P. intermedia* is associated more clearly with disease. The confusion in the literature obviously is due to difficulties in separating these two species from each other using biochemical identification methods. In the present study, the prevalence of *P. nigrescens* was the highest in all age groups and remained stable throughout the age groups. Our results are concordant with a similar study conducted by Ooshima et al. [15], whose report showed that *P. nigrescens* was detectable in the oral cavity of healthy children as early as 4 years and their detection rates increased with age reaching to a frequency of 73% at the age of 14. Okada et al. [6] also reported a 45.8% detection rate for *P. nigrescens* in children between ages 2 and 12 years. Thus, it is conceivably plausible that *P. nigrescens* is indeed a common member of the oral microbial flora of healthy children. However, *P. intermedia*, like *T. forsythensis*, were found in low proportions of the children included in our study. Interestingly, in our present study, while 10% of children in age group less than 10 years were positive for *P. intermedia*, this pathogen was not detected in any of the children in the same age group of the Ooshima's study but the proportion of children positive for *P. intermedia* in the older age groups was similar. Other studies have shown that this organism has been isolated from both healthy and diseased sites [16].

T. forsythensis, appears to occur in the same relatively low number of children, as does *P. intermedia*, particularly in the age below 10 years of age. Thus, our finding on this organism is at variance with that of Ooshima et al. [15] in that, in this age group, only 5% of the children harbored *T. forsythensis* compared with 43% in theirs. The age group with highest number of children harboring the organism, in our study, was the 13–15 years group. This probably supports earlier findings which claimed that *T. forsythensis* is a common member of the oral microbial flora of healthy children [15].

Unlike in the report of Ooshima et al. [15], *P. gingivalis* was found in much lower proportions of the children in our study. The prevalence of *P. gingivalis*, and to some extent, *P. intermedia*, and *T. forsythensis* may be transient and their colonization in periodontally healthy children might be a rare event. Although colonization does not necessarily induce an infection that causes destruction of the periodontium, continuous presence is likely a prerequisite for these three bacteria. In contrast, the level of *A. actinomycetemcomitans* and *P. nigrescens* present in the subgingival site may be a key factor for the prediction of periodontitis in children.

Studies have shown that their presence in the saliva is a reflection of their colonization of the subgingival and supragingival sites [17,18]. The act of stimulation by chewing a piece of paraffin wax allows the still-attached bacteria or clumps of microbes to loosen from the oral biofilms into saliva. In fact, some studies have demonstrated the superiority of saliva over pooled subgingival samples for the detection of some periodontal pathogens [19,20]. Multiplex PCR have been developed to identify these pathogens in subgingival plaques but recent studies have shown that modified protocol involving treating saliva with cation-chelating resin produced comparable PCR results [12,21].

Prevalence study of this nature using qualitative molecular amplification (PCR) methodologies is not without its limitations, especially when comparing the prevalence results with some published data based on less sensitive conventional cultural methods with their confounding taxonomy and ambiguity of phenotype database. In addition, the density of each of the pathogens which may be relevant to the degree of carriage of these bacteria in the mouth of healthy children could not be determined by the qualitative nucleic acid testing employed in our study.

In conclusion, the present study shows that some periodontal pathogens are present in childhood particularly from the age of 6 years upwards. It appears that *P. gingivalis* rarely colonizes the oral cavity of children and that the occurrence of *A. actinomycetemcomitans* decreases with age in childhood. Monitoring the carriage rate of periodontal pathogens in childhood may be beneficial to designing preventive strategies for the control of periodontal diseases/infections.

Conflict of interest statement

There are no competing of conflicting interest.

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